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Variations in the stimulus salience of cocaine reward influences drug-associated contextual memory

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Abstract

Drugs of abuse act as reinforcers because they influence learning and memory processes resulting in long-term memory of drug reward. We have previously shown that mice conditioned by fixed daily dose of cocaine (Fix-C) or daily escalating doses of cocaine (Esc-C) resulted in short- and long-term persistence of drug memory, respectively, suggesting different mechanisms in acquisition of cocaine memory. The present study was undertaken to investigate the differential contribution of N-methyl-D-aspartate receptor (NMDAR) subunits in the formation of Fix-C and Esc-C memory in C57BL/6 mice. Training by Esc-C resulted in marked elevation in hippocampal expression of Grin2b mRNA and NR2B protein levels compared to training by Fix-C. The NR2B-containing NMDAR antagonist ifenprodil had similar attenuating effects on acquisition and reconsolidation of Fix-C and Esc-C memory. However, the NMDAR antagonist MK-801 had differential effects: a) higher doses of MK-801 were required for post-retrieval disruption of reconsolidation of Esc-C memory than Fix-C memory, and b) pre-retrieval MK-801 inhibited extinction of Fix-C memory but it had no effect on Esc-C memory. In addition, blockade of NMDAR downstream signaling pathways also showed differential regulation of Fix-C and Esc-C memory. Inhibition of neuronal nitric oxide synthase (nNOS) attenuated acquisition and disrupted reconsolidation of Fix-C but not Esc-C memory. In contrast, the mitogen-activating extracellular kinase (MEK) inhibitor SL327 attenuated reconsolidation of Esc-C but not Fix-C memory. These results suggest that NMDAR downstream signaling molecules associated with consolidation and reconsolidation of cocaine-associated memory may vary upon changes in the salience of cocaine reward during conditioning.

Keywords

cocaine; place preference; addiction; NR2B; reconsolidation

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AUTHOR CONTRIBUTIONS

SL and YI were responsible for the study concept, design and interpretation of findings. SL performed all data collection and analysis. SL drafted the manuscript while YI provided critical revision for intellectual content. Both authors critically reviewed content and approved final version for publication.

The authors declare no conflict of interest.

INTRODUCTION

The role of learning and memory in the reinforcing effects of addictive drugs continues to garner much attention. Persistent drug-seeking behavior and the inability to extinguish such maladaptive behavior develop when drugs of abuse exert control over neural substrates and signaling pathways that encode long-term memory (LTM) (Hyman *et al.* 2006). Recently, disruption of persistent drug memory has taken the forefront as a possible treatment strategy for addiction. Memory reconsolidation is the process whereby previously consolidated memories, upon retrieval, become labile and are thereby susceptible to disruption. A number of studies have shown that cocaine-associated memories are vulnerable to disruption upon retrieval of such memories (Miller & Marshall, 2005; Kelley *et al.* 2007; Tronson & Taylor, 2013).

The conditioned place preference (CPP) paradigm, which employs the principles of Pavlovian learning, can model learning and memory processes pertinent to addictive behavior (White & Carr, 1985). One caveat in CPP studies is the use of a fixed daily dose of the drug reinforcer during training. Given that the transition from drug use to drug addiction involves escalation in drug intake, we posit that investigation of the outcome of escalating doses of cocaine during conditioning is relevant to the human practice of drug use and the development of addictive behavior. Our laboratory (Itzhak & Anderson, 2012) has shown that conditioning by escalating doses of cocaine (Esc-C) produced higher magnitude and more persistent CPP than conditioning by fixed daily doses of cocaine (Fix-C). This phenomenon was not dose-dependent but rather schedule-dependent; these results were recently confirmed by others (Conrad *et al.* 2013).

While some studies have used drug self-administration to investigate escalation in drug exposure, results have shown only a modest escalation in drug self-administration; about 1.2–1.5-fold increase over a two-week period (Ahmed & Koob, 1998; Perry *et al.* 2006; Anker *et al.* 2012). However, others interpreted these results as a correlate of long access duration to the drug and not escalated drug intake (Knackstedt & Kalivas, 2007). We posit that investigation of mechanisms involved in formation of drug memory can be better modeled in the CPP paradigm by introducing significant changes in the stimulus salience of the drug reward which apparently facilitates contextual learning.

The *N*-methyl-D-aspartate receptor (NMDAR) plays a central role in synaptic plasticity and learning and memory processes (Collingridge, 1987). With respect to cocaine effects, the NMDAR antagonist MK-801 disrupted acquisition, expression and reconsolidation of cocaine-associated memory (Kelley *et al.* 2007; Alaghband & Marshall, 2013). Functional NMDARs typically exists as heterotetramers between two compulsory NR1 subunits and two modulatory NR2 subunits (NR2A-D) (Zhou, 2009). The NR2 subunits of NMDARs are thought to have a major role in learning and memory (Furukawa *et al.* 2005). The abundance of specific NR2 subunit appears to be developmentally regulated where NR2B subunits predominate during early brain development while NR2A levels increase progressively with development (Yashiro & Philpot, 2008). The key functional properties of NMDARs are thought to be mediated by the particular NR2 subunits that comprise the receptor channel (Monyer *et al.* 1994). Indeed, compared to NR2A-containing NMDARs, NR2B-containing

NMDARs display longer decay time constant (Cull-Candy & Leszkiewicz, 2004) and carry greater calcium current per unit charge (Sobczyk *et al.* 2005). Additionally, it has been reported that NR2A- and NR2B-containing NMDARs are coupled to different downstream signaling pathways (Chen *et al.* 2007).

One downstream effector of calcium influx through NMDAR is neuronal nitric oxide synthase (nNOS), which catalyzes the production of nitric oxide (NO) (Brenman & Bredt, 1997). Calcium-dependent nNOS is linked via the post synaptic density protein PSD-95 to the NR2 subunit of the NMDAR; thus its selective localization renders it functionally coupled to the NMDAR (Christopherson *et al.* 1999; Sattler *et al.* 1999). Relevant to cocaine effects, NO has been implicated as a major contributor to the initiation and maintenance of cocaine CPP and behavioral sensitization (Bhargava & Kumar, 1997; Itzhak *et al.* 1998; Balda *et al.* 2006). Another downstream molecule activated by calcium entry through NMDAR is extracellular signal-regulated kinase (ERK) (Sweatt, 2001). One of many regulators of the ERK signaling cascade is RasGRF1, a Ras-specific GDP/GTP exchange factor (GEF) and Ras activator that specifically binds the NR2B subunit of NMDARs. Hence, NR2B-containing NMDARs are mediators of the NMDAR-dependent ERK signaling via Ras-Raf-MEK-ERK signaling (Krapivinsky *et al.* 2003). With respect to cocaine effects, inhibition of mitogen-activating extracellular kinase (MEK), the ERK kinase, has been shown to disrupt cocaine-associated contextual memory (Miller & Marshall, 2005; Valjent *et al.* 2006).

Because the NMDAR and its downstream molecules have a role in learning and memory and cocaine effects, this study was undertaken to elucidate the involvement of NMDAR subunits and downstream signaling pathways in acquisition and reconsolidation of Fix-C and Esc-C memory.

METHODS AND MATERIALS

Subjects

Male C57BL/6J mice (8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were housed in groups of 5/cage with food and water ad-libitum and were acclimatized to the vivarium for one week before experiments began. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and was approved by the University of Miami Animal Care and Use Committee.

Drugs

Cocaine HCl and (+)-MK-801 hydrogen maleate (dizocilpine) were dissolved in saline (0.9% NaCl). The NR2B antagonist ifenprodil [α -(4-Hydroxyphenyl)- β -methyl-4-benzyl-1-piperidine-ethanol(+)-tartrate salt] was dissolved in distilled water. Traxoprodil [(1S,2S)-1-(4-hydroxy-phenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol], another NR2B-containing NMDAR antagonist (Chenard *et al.* 1995), and the nNOS inhibitor 7-nitroindazole (7-NI) were dissolved in a 1:3:6 mixture of DMSO, polyethylene glycol and distilled water, respectively. The MEK inhibitor SL327 was dissolved in 40% DMSO

(Atkins *et al.* 1998). All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA). Drugs and vehicles were administered intraperitoneally (i.p.) in a volume of 0.1ml/10g.

Conditioning procedure

Place preference was monitored using custom-designed Plexiglas cages (Opto-Max Activity Meter v2.16; Columbus Instruments) as previously described (Itzhak and Anderson, 2012; Liddie *et al.* 2012). The training context consisted of two compartments. One compartment had black and white striped walls and a white floor covered with stainless steel grid; the other compartment had black walls and smooth black floor. Each compartment was scanned by 7 infrared beams. A null zone 8 cm wide was assigned at the interface of the two compartments to ensure that only full entry into each compartment is registered as 'real' time spent in each zone. On the first day, mice were habituated (15min) to the training context; preconditioning compartment-preference/aversion was determined. Pre-conditioning average times spent in the black, striped and null zones during 1200 seconds were 456 ± 12 , 516 ± 13 and 217 ± 9 seconds, respectively. Half the subjects showed slight preference for one side or the other. Accordingly, mice were paired with cocaine in the less preferred compartment. Although this may be viewed as a biased design, half of the mice were paired with cocaine in the black compartment and the other half were paired with cocaine in the black and white-striped compartment making this design 'partially biased'. Following habituation, mice were conditioned over 4 days by a) 11.25mg/kg (Fix-C) or b) 3, 6, 12 and 24mg/kg; one dose per day (Esc-C) as we previously described (Itzhak & Anderson, 2012). Doses were chosen to control for total amount of cocaine administered over 4 days. Post-conditioning average time spent in the null zone during 1200 seconds CPP test was 192 ± 7 seconds. Likewise time spent in the null zone following pharmacological treatments, pre- and post-CPP, was not significantly different than vehicle treatment (201 ± 9 seconds).

Experiment 1: Contribution of NMDAR subunits in formation of Fix-C and Esc-C memory

To determine whether NMDAR subunits were differentially regulated in mice conditioned by Fix-C and Esc-C (Itzhak & Anderson, 2012) bilateral hippocampus was dissected 24h after conditioning and subsequently analyzed. We performed quantitative real-time polymerase chain reaction (qPCR) analysis ($n=4$ mice/group) using custom designed qPCR arrays (Qiagen) and immunoblot analyses ($n=3-4$ mice/group) for NR1 (1:4000; 5704S; Cell Signaling), NR2A (1:4000; 4205S; Cell Signaling), NR2B (1:4000, 06-600; Millipore) and β -tubulin (1:40000; 05-559; Millipore). Detailed qPCR and Western blot procedures are available in the Supplementary Materials and Methods. We focused on the hippocampus because of its role in spatial/contextual memory. Experimental groups for Fix-C and Esc-C included the following: **Coc-Paired**: saline was given in one compartment at mornings and cocaine in the other compartment 3h later. **Coc-Unpaired**: saline was given in one compartment at mornings and 3h later mice were re-exposed to the conditioning apparatus in the absence of drug; cocaine was administered (30min later) in the home cage. **Saline-Paired**: saline given in both compartments. For qPCR the saline, paired and unpaired (Fix-C and Esc-C) groups were analyzed whereas for western blot analyses, only the saline controls and paired groups of Fix-C and Esc-C were analyzed.

Experiment 2: Acquisition of Esc-C and Fix-C memory

We investigated the roles of the NR2B subunit and NO-signaling in acquisition of Fix-C and Esc-C memory. Thirty minutes prior to each cocaine session, mice received a) vehicle, b) ifenprodil (10mg/kg) or c) 7-NI (25mg/kg). Mice were tested for place preference (15min) 72hr later (day 8).

Experiment 3: Reconsolidation of Fix-C and Esc-C memory

3a) Effect of the NMDAR antagonist MK-801—We then investigated the effect of MK-801 on reconsolidation of Fix-C and Esc-C memory. Following conditioning, on day 8, mice (n=6–10/group) received MK-801 (0.1 or 0.3mg/kg) pre- or post-retrieval of place memory. For pre-retrieval, drug was given 30 min prior to CPP expression test (15min) and for post-retrieval drug was given following a 6 min CPP expression test. Mice were then tested for place preference expression after 4 days.

3b) Effect of NR2B subunit antagonists ifenprodil and traxoprodil—Next, we investigated the effects of ifenprodil and traxoprodil on Fix-C and Esc-C memory reconsolidation. On day 8, mice were re-exposed to the conditioning apparatus for 6min (memory retrieval by a short CPP test) and immediately thereafter they received ifenprodil (10mg/kg) or traxoprodil (10mg/kg). To test whether subsequent reductions in CPP following ifenprodil treatment was due to disruption of memory reconsolidation, ifenprodil was administered a) in the home cage in the absence of memory retrieval or b) 6h after memory retrieval (outside the reconsolidation window). Mice were then tested for place preference expression 4 days following the first memory retrieval session.

3c) Effect of the nNOS inhibitor 7-NI and the MEK inhibitor SL327—To investigate the role of NMDAR downstream signaling in memory reconsolidation, the effects of the nNOS inhibitor 7-NI and the MEK inhibitor SL327 were investigated. Mice were conditioned by Fix-C and Esc-C schedules and after 72h mice were re-exposed to the conditioning apparatus for 6 min (memory retrieval by a short CPP test). Vehicle, 7-NI (25mg/kg) or SL327 (30mg/kg) were administered immediately thereafter. Mice were then tested for place preference expression 4–7 days following the first memory retrieval session.

Data Analysis

Data for behavioral testing were analyzed using two-way ANOVA [(group x time) or (MK-801 dose x time; experiment 3)] followed by Student-Newman-Keuls *post hoc* test. qPCR data were analyzed using 2^{-CT} method as described by Livak and Schmittgen (2001). Differences in expression levels in qPCR and western blot analyses were analyzed by one-way ANOVA. Student's t-test was also used to compare differences between two groups. Data analysis was done using Sigma Stat version 3.1 (Systat Software Inc.).

RESULTS

Experiment 1: Contribution of NMDAR subunits in formation of Fix-C and Esc-C memory

Mice that received cocaine paired to the training context demonstrated significant place preference while mice that received either saline injections or cocaine unpaired to the

training context did not (Fig. 1A; day 6). Two-way ANOVA showed there was a significant group effect [for Fix-C: $F_{(2,48)}=7.422$; $p=0.002$; for Esc-C: $F_{(2,54)}=12.258$; $p<0.001$]; a significant time effect [for Fix-C: $F_{(1,48)}=10.201$; $p=0.002$; for Esc-C: $F_{(1,54)}=30.231$; $p<0.001$] and a significant group x time interaction [for Fix-C: $F_{(2,48)}=4.323$; $p=0.019$; for Esc-C: $F_{(2,54)}=8.898$; $p<0.001$]. A comparison of the magnitude of CPP on day 6 between Esc-C-paired and Fix-C-paired found that Esc-C showed significantly higher CPP than Fix-C ($t=2.355$; $p=0.031$) – thus confirming our previous findings (Itzhak & Anderson, 2012).

With respect to NMDAR subunit mRNA expression, one-way ANOVA comparing Grin2b expression among the saline control group, and the Fix-C and Esc-C paired and unpaired groups showed a significant group effect [$F_{(4,15)}=6.473$; $p=0.003$; Fig. 1D inset]. Grin2b was significantly up-regulated in the paired Esc-C group compared to all other groups; no significant difference in Grin2b expression between the unpaired groups (Fix-C and Esc-C) was observed (Fig. 1D inset). Levels of Grin1 and Grin2a mRNA were unchanged across all groups (data not shown).

Because the unpaired conditions showed no significant differences in Grin2b mRNA expression, western blot analyses were conducted using only the paired conditions (Fix-C and Esc-C). Western blot analyses of NR2A, NR2B and the compulsory NR1 subunits were carried out. NR2B was significantly up-regulated in Esc-C compared to Fix-C and saline-treated mice. One-way ANOVA revealed an overall significant group effect [$F_{(2,8)}=12.664$; $p=0.003$]. Figure 1D shows an approximate 2.5-fold increase in total NR2B in the hippocampus of Esc-C compared to Fix-C and approximately 5-fold increase compared to saline-treated mice. Student's t-test showed Fix-C was also significantly up-regulated approximately 2.3-fold over saline-treated mice ($t=2.684$; $p=0.044$). There were no significant differences in expression levels of NR1 and NR2A across the groups although there appeared to be a trend toward reduction in NR2A where saline>Fix-C>Esc-C (Fig. 1B, C).

Experiment 2: Acquisition of Esc-C and Fix-C memory

We investigated whether systemic administration of the NR2B-containing NMDAR antagonist ifenprodil would attenuate the acquisition of Fix-C and Esc-C memory. Two-way ANOVA analysis of the effect of ifenprodil treatment on cocaine-associated memory expression showed that for Esc-C, there was a significant group effect [$F_{(1,30)}=6.678$; $p=0.015$], a significant time effect (test day) [$F_{(1,30)}=121.882$; $p<0.001$] and a significant group x time interaction [$F_{(1,30)}=7.586$; $p=0.01$]. For Fix-C, there was a significant time effect [$F_{(1,28)}=46.351$; $p<0.001$], however, there was no significant group effect [$F_{(1,28)}=1.894$; $p=0.18$] and no significant group x time interaction [$F_{(1,28)}=4.001$; $p=0.055$]. *Post hoc* analyses showed that administration of ifenprodil 30 min before each cocaine administration session significantly reduced the expression of place preference in both Esc-C ($p=0.004$) and Fix-C ($p=0.024$) compared to saline-treated animals (Fig 2B, C; day 8).

We next investigated whether inhibition of nNOS influences acquisition of Esc-C memory. Pretreatment with the nNOS inhibitor 7-NI blunted the development of place preference for mice conditioned by Fix-C [$F_{(1,26)}=4.908$; $p=0.036$; group effect] but not Esc-C ($p=0.995$)

(Fig 2D, E), suggesting NO-independent formation of Esc-C memory. There was a significant time effect for both Fix-C [$F_{(1,26)}=64.991$; $p<0.001$] and Esc-C [$F_{(1,26)}=150.662$; $p<0.001$], however, there was no significant group x time interaction for neither Fix-C nor Esc-C groups ($p>0.05$).

Experiment 3: Reconsolidation of Fix-C and Esc-C memory

Administration of MK-801 before the CPP test (pre memory retrieval) prevented a reduction in place preference for Fix-C [$F_{(1,48)}=8.904$; $p=0.004$; group effect] but it had no effect on Esc-C (Fig 3B,C). For Fix-C, there was a significant time effect [$F_{(2,48)}=48.437$; $p<0.001$] and a significant group x time interaction [$F_{(2,48)}=3.246$; $p=0.048$]. For Esc-C, there was a significant time effect [$F_{(2,33)}=85.508$; $p<0.001$] but no significant group x time interaction ($p>0.05$). The results suggest that the first exposure to the CPP context may elicit extinction in the Fix-C but not Esc-C group and MK-801 inhibited the extinction in the Fix-C group.

In the next experiment MK-801 was administered 6 min following the first CPP test (post memory retrieval). A dose of 0.1mg/kg MK-801 attenuated subsequent expression of Fix-C. Two-way ANOVA showed a significant time effect [$F_{(2,39)}=43.151$; $p<0.001$] but no significant group effect nor group x time interaction ($p>0.05$). *Post hoc* analysis showed that on day 12, MK-801-treated animals showed reduced CPP compared to saline-treated mice ($p=0.012$), suggesting that MK-801 disrupted memory reconsolidation. With respect to Esc-C, two-way ANOVA comparing the dose effect of MK-801 on disruption of reconsolidation showed a significant group (MK-801 dose) effect [$F_{(2,66)}=5.747$; $p=0.005$], a significant time effect [$F_{(2,66)}=83.735$; $p<0.001$] but no significant group x time interaction. Results suggest that while a low dose (0.1mg/kg) MK-801 was sufficient to disrupt Fix-C memory reconsolidation, a higher dose of MK-801 (0.3mg/kg) was required for disruption of Esc-C memory reconsolidation (Fig. 3E; day 12).

In all subsequent reconsolidation experiments drugs were administered following a 6 min CPP test (post memory retrieval). For both Fix-C and Esc-C, the 6 min exposure to the context was sufficient to express the acquisition of CPP (Pre-CPP vs Retrieval; $p<0.001$; Fig 4). Ifenprodil (group effect) significantly disrupted reconsolidation of both Fix-C and Esc-C memory [for Fix-C: $F_{(1,48)}=4.123$; $p=0.048$; for Esc-C: $F_{(1,39)}=11.832$; $p=0.001$; Fig 4B, C]. There was a significant time effect [for Fix-C: $F_{(2,48)}=31.171$; $p<0.001$; for Esc-C: $F_{(2,39)}=25.697$; $p<0.001$] but no significant group x time interaction. Administration of ifenprodil either in the home cage in the absence of memory retrieval or 6h after memory retrieval had no effect on Esc-C CPP (Fig 4D), suggesting that ifenprodil disrupted memory reconsolidation. To further validate the involvement of NR2B in Fix-C and Esc-C memory reconsolidation, the effects of traxoprodil was investigated. Traxoprodil is another NR2B receptor antagonist which has previously been tested in clinical trials for the treatment of depression (Preskorn *et al.* 2008), dyskinesia and Parkinsonism (Nutt *et al.* 2008). Administration of traxoprodil following memory retrieval significantly reduced subsequent expression of CPP in both Fix-C and Esc-C (Fig 4E, F). Two-way ANOVA showed a significant group effect for Esc-C [$F_{(1,42)}=9.466$; $p=0.004$] but not Fix-C [$F_{(1,42)}=2.555$; $p=0.117$]. There was a significant time effect for both Esc-C [$F_{(2,42)}=75.191$; $p<0.001$] and Fix-C [$F_{(2,42)}=35.280$; $p<0.001$] but no significant group x

time interaction in either case. *Post hoc* analysis showed that traxoprodil-treated mice displayed significantly lower CPP scores than vehicle-treated mice for both Esc-C ($p<0.001$) and Fix-C ($p=0.005$) on day 12.

We then sought to isolate the contribution of different signaling molecules downstream of the NMDAR in cocaine-memory reconsolidation. Post-retrieval administration of the nNOS inhibitor 7-NI reduced the magnitude of subsequent CPP that was acquired by Fix-C but not Esc-C schedule (Fig. 5B, C), suggesting disruption of Fix-C [$F_{(1,45)}=6.558$; $p=0.014$] but not Esc-C [$F_{(1,48)}=0.0166$; $p=0.898$] memory reconsolidation. There was a significant time effect for Fix-C [$F_{(2,45)}=42.089$; $p<0.001$] and Esc-C [$F_{(1,48)}=29.907$; $p<0.001$] but no significant group x time interaction. The disruption of Fix-C memory reconsolidation by 7-NI is consistent with our previous studies (Itzhak & Anderson, 2007).

We then investigated the involvement of ERK in reconsolidation of Fix-C and Esc-C memory. The ERK kinase (MEK) inhibitor SL327 disrupted Esc-C but not Fix-C memory reconsolidation (Fig 5D, E). Two-way ANOVA showed no significant group effect for Esc-C [$F_{(1,36)}=3.904$; $p=0.056$] nor Fix-C [$F_{(1,51)}=0.873$; $p=0.354$]. However, there was a significant time effect [for Esc-C: $F_{(2,36)}=49.884$; $p<0.001$; for Fix-C: $F_{(2,51)}=63.774$; $p<0.001$] and a significant group x time interaction for Esc-C [$F_{(2,36)}=3.727$; $p=0.034$] but not for Fix-C [$F_{(2,51)}=0.344$; $p=0.71$]. *Post hoc* analysis found a significant reduction in CPP on day 12 for Esc-C ($p=0.014$) but not Fix-C ($p>0.05$). Taken together, these results suggest that Fix-C memory engages NO signaling while Esc-C memory may bypass the dependence on NO and engages the NMDAR-dependent ERK signaling pathway.

DISCUSSION

Previous studies have indicated the significance of variations in the daily schedule of cocaine within the conditioning phase, rather than the dose of cocaine, on the development of drug-associated memory (Itzhak & Anderson, 2012; Conrad *et al.* 2013). While conditioning by fixed daily doses of cocaine resulted in relatively low magnitude of place preference and rapid extinction, conditioning by escalating doses of cocaine resulted in higher magnitude of place preference, and resistance to extinction by re-exposure to nonreinforced context (Itzhak & Anderson, 2012; Liddie *et al.* 2012). Given these observations, the present study was undertaken to investigate whether Fix-C and Esc-C memory engaged different neural pathways in the formation and reconsolidation of cocaine-associated memory.

Since we were interested in the effect of cocaine-context-associated learning following Fix-C and Esc-C schedules, we focused on the expression levels of NMDAR subunits. The NMDAR is thought to mediate synaptic plasticity and learning and memory (Collingridge, 1987) and different subunits are coupled to specific downstream signaling molecules (Chen *et al.* 2007). Therefore, the investigation of NMDAR subunits expression could elucidate differences between Fix-C and Esc-C memory (Itzhak & Anderson, 2012). We found that conditioning by Esc-C results in increased hippocampal expression of both Grin2b mRNA and NR2B protein, suggesting an induction of both transcription and translation of NR2B subunits of the NMDAR. However, in the Fix-C group we did not detect an increase in

Grin2b mRNA and only an increase in NR2B protein was observed. The lack of increase in Grin2b mRNA in the Fix-C group is unclear but studies have shown varied correlation between mRNA and protein levels; a result of biological factors which influence transcription and translation processes (Nie *et al.* 2006). Other studies have shown NR2B protein up-regulation in rat nucleus accumbens following repeated cocaine administration (Huang *et al.* 2009) and in rat hippocampus following morphine-induced CPP (Ma *et al.* 2006). Interestingly, however, we observed that the increase in expression levels of NR2B in mice conditioned by the Esc-C schedule was higher than in mice conditioned by the Fix-C schedule. This finding may be relevant to the strength of Esc-C memory compared to Fix-C memory.

Evidence suggests that the NR2B subunit of NMDAR has potential to carry greater calcium current per unit charge (Sobczyk *et al.* 2005) which may confer a greater influence on downstream signaling cascades that affect synaptic plasticity and learning and memory. For example, rats over-expressing NR2B in the cortex and hippocampus showed improved performance in a number of learning and memory tasks (Wang *et al.* 2009), while pharmacological or genetic blockade of the NR2B subunit in the cingulate cortex of mice impaired the formation of contextual fear memory (Zhao *et al.* 2005). Additionally, recent reports suggest that repeated cocaine administration generates silent synapses (Huang *et al.* 2009). Silent synapses contain higher levels of NR2B-containing NMDARs compared to neighboring synapses and are capable of undergoing rapid metaplasticity to strengthen synapses (Lee *et al.* 2010). Hence, it is plausible that the increased expression of NR2B in the present study may have contributed to the development of a more 'stable' Esc-C memory compared to Fix-C. A trend toward a reduction in levels of NR2A (Fig 1C) may indicate a switch in NMDAR subunit composition in Esc-C mice where NR2B replaces NR2A.

Because of the changes in expression of NR2B, we investigated whether selective antagonism of NR2B-containing NMDAR could disrupt cocaine-associated memory. The NR2B antagonist ifenprodil a) attenuated acquisition and b) reduced subsequent place preference expression when administered following memory retrieval in both Fix-C and Esc-C groups. The former result is in accordance with others who showed that ifenprodil prevented the development of cocaine (Kiraly *et al.* 2011) and morphine (Ma *et al.* 2006) CPP following fixed administration schedules. The latter result was likely due to disruption of memory reconsolidation since administration of ifenprodil either a) following retrieval but outside the reconsolidation window or b) in the absence of memory retrieval, had no effect on subsequent CPP expression (Fig. 4D). The absence of place preference in the acquisition and reconsolidation experiments was not due to an aversive influence of ifenprodil since a dose of 10mg/kg ifenprodil is neither rewarding nor aversive (Suzuki *et al.* 1999). While we cannot completely rule out extinction learning, it is feasible to assume that ifenprodil disrupted memory reconsolidation since we have previously shown that mice conditioned by Esc-C do not exhibit extinction with few unreinforced exposures to the CPP context (Itzhak & Anderson, 2012). We corroborated our findings by demonstrating that another NR2B antagonist traxoprodil was similarly effective at disrupting the reconsolidation of both Fix-C and Esc-C memory. Taken together, we demonstrate that

NR2B-containing NMDARs play a behaviorally significant role in the development and reconsolidation of cocaine-associated memory independent of the schedule of conditioning.

However, the NMDAR antagonist MK-801 had differential effects on reconsolidation of Fix-C and Esc-C memory and it appears that the dependence of NMDAR in the process of memory reconsolidation is temporally mediated. First, post-retrieval administration of low dose MK-801 (0.1mg/kg) disrupted Fix-C memory reconsolidation while a higher dose (0.3mg/kg) of the NMDAR antagonist was required to disrupt Esc-C memory reconsolidation. The increased expression of NR2B subunit in the Esc-C group, relative to the Fix-C group, may be associated with facilitated calcium entry (Sobczyk *et al.* 2005) and therefore a higher dose of the NMDAR antagonist was required to inhibit downstream signaling molecules involved in memory reconsolidation. Second, pre-retrieval administration of MK-801 a) prevented extinction of Fix-C CPP since saline-treated mice showed reduced place preference while CPP was maintained in MK-801-treated mice and b) had no effect on Esc-C CPP (Fig. 3). This finding implies that the Fix-C group, but not the Esc-C group, was undergoing extinction learning (reduction in preference for the cocaine-paired compartment), which was prevented by MK-801. This premise is supported by our previous observations that Fix-C memory is more susceptible to extinction compared to Esc-C memory (Itzhak & Anderson, 2012). Similar to the current observations, a) post-retrieval antagonism of the NMDAR disrupted reconsolidation of object recognition (Akirav & Mamoun, 2006) and odor-reward memory (Torrás-García *et al.* 2005) and b) pre-retrieval antagonism of NMDAR prevented extinction of conditioned freezing response (Ben Mamou *et al.* 2006). Other studies demonstrated that pre- but not post-retrieval systemic administration of MK-801 prevented memory reconsolidation in a conditioned reinforcement behavioral task (Milton *et al.* 2008). This discrepancy in temporal-dependence of MK-801 may be due to differences in behavioral paradigms which investigate instrumental (self-administration) versus non-operant memory tasks.

Further differences between Fix-C and Esc-C memory are associated with NMDAR downstream signaling molecules. Since the activity of nNOS is coupled to calcium influx through the NMDAR (Christopherson *et al.* 1999; Sattler *et al.* 1999) we investigated whether the observed differences in behavioral phenotype between Fix-C and Esc-C was a function of nNOS involvement. First, the nNOS inhibitor 7-NI prevented the formation of Fix-C memory but not Esc-C memory (Fig 2). Second, reconsolidation of Fix-C but not Esc-C memory was disrupted by the nNOS inhibitor. The latter confirms our previous studies on the dependency of Fix-C memory on NO signaling (Itzhak & Anderson, 2007) but reveals now a NO-independent signaling mechanism for Esc-C memory. This differential effect of nNOS inhibition may be due to the recruitment of additional signaling pathways in response to the more salient conditioning schedule (Esc-C). Hence, although nNOS could potentially be activated in response to training by Esc-C, the activation of other signaling pathways may overshadow the involvement of nNOS.

Calcium influx through NMDARs has the potential to activate several calcium-dependent signaling pathways; for instance, the NMDAR-RasGRF1-MEK-ERK pathway. ERK is an important regulator of neuronal plasticity, long-term potentiation (LTP) and LTM formation (Krapivinsky *et al.* 2003). Since RasGRF1 specifically binds the NR2B subunit of the

NMDAR, it couples the activity of ERK with NR2B-containing NMDARs (Krapvinisky *et al.* 2003). Studies have shown that associative learning leads to an up-regulation of ERK in the hippocampus and inhibition of the ERK kinase MEK, disrupted the formation of fear memory (Atkins *et al.* 1998). Furthermore, it has been suggested that NR2A- and NR2B-containing NMDARs are coupled to different signaling pathways; activation of NR2A increased brain derived neurotrophic factor (BDNF) expression while activation of NR2B-containing NMDARs led to phosphorylation of ERK (Chen *et al.* 2007).

Because the CPP paradigm involves associative learning and we observed differential expression of NR2B subunits, we investigated whether reduced ERK activation via MEK inhibition would disrupt memory reconsolidation of Fix-C and Esc-C. We found that MEK inhibition had differential effects on Fix-C and Esc-C memory where reconsolidation of Esc-C but not Fix-C memory was disrupted by SL327. Because NMDAR-dependent ERK phosphorylation is coupled to NR2B, inhibition of MEK is expected to have a greater effect on NMDAR-dependent ERK signaling where levels of NR2B are substantially elevated; that is, in Esc-C group. The modest increase in NR2B in the Fix-C group may have been sufficient to allow sensitivity to ifenprodil treatment but insufficient to promote a robust NMDAR-dependent activation of ERK. Contrary to our findings where MEK inhibition had no effect on Fix-C memory reconsolidation, bilateral injections of U0126, another MEK inhibitor, into nucleus accumbens core disrupted expression and reconsolidation of Fix-C CPP (Miller & Marshall, 2005). This apparent discrepancy may be due to differences in the route of inhibitor administration (intracerebral vs intraperitoneal) and duration of conditioning (9 days vs 4 days). Others have reported that systemic administration of SL327 in a fixed-dose cocaine CPP paradigm effectively disrupted memory reconsolidation only when memory retrieval occurred by pairing the context with cocaine (Valjent *et al.* 2006). This finding suggests that the conditioned stimulus alone (CPP context) was insufficient to engage MEK signaling in Fix-C but instead it required the much stronger unconditioned stimulus (cocaine). Thus in the present study, SL327 may have been ineffective at disrupting Fix-C memory reconsolidation because subjects were exposed to the CPP context alone. However, the MEK inhibitor was effective in disrupting reconsolidation of Esc-C memory. This finding suggests that context re-exposure of Esc-C (but not Fix-C) mice was sufficient to invoke 'strong memory' of drug-context association which engaged MEK-signaling.

While the nNOS signaling pathway may also be activated in response to training by Esc-C, it appears that other signaling pathways including NMDAR-MEK-ERK signaling plays a more behaviorally significant role in the development of Esc-C CPP. Additionally, though both NO-cGMP-PKG and MEK signaling pathways converge at the level of ERK (Ota *et al.* 2008) we posit that the contribution of each pathway to drug memory is dependent on cocaine conditioning schedule.

We focused on identifying molecular changes in the hippocampus because of its role in contextual memory. While our results lend credence to the involvement of the hippocampus with respect to changes in NR2B receptor subunit expression, downstream NMDAR signaling molecules in other brain such as nucleus accumbens, amygdala and prefrontal cortex may also be involved in the behavioral effects we observed.

In summary, we show that the salience of cocaine reward influences memory strength by engaging different neural pathways. The NR2B subunit of NMDARs and MEK-associated signaling appears to have a major role in drug memory acquired by escalating dose of cocaine, while NMDAR and NO-associated signaling appear to be involved in drug memory encoded by the Fix-C schedule. Given that drug addiction is associated with escalation in drug use, we posit that different schedules of drug exposure may result in differential strength in drug memory which could be relevant to the severity of addiction. Our data opens the door to further investigations into the effects of drug memory strength and its differential susceptibility to pharmacological manipulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Ahmed SH, Koob GF. Transition from moderate to excessive drug intake: change in hedonic set point. *Science*. 1998; 282:298–300. [PubMed: 9765157]
- Akirav I, Maroun M. Ventromedial prefrontal cortex is obligatory for consolidation and reconsolidation of object recognition memory. *Cereb Cortex*. 2006; 16:1759–1765. [PubMed: 16421330]
- Alagband Y, Marshall JF. Common influences of non-competitive NMDA receptor antagonists on the consolidation and reconsolidation of cocaine-cue memory. *Psychopharmacology*. 2013; 226:707–719. [PubMed: 22829432]
- Anker JJ, Baron TR, Ziebnik NE, Carroll ME. Escalation of methamphetamine self-administration in adolescent and adult rats. *Drug Alcohol Depend*. 2012; 124:149–153. [PubMed: 22305912]
- Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, Sweatt JD. The MAPK cascade is required for mammalian associative learning. *Nat Neurosci*. 1998; 7:602–609. [PubMed: 10196568]
- Balda MA, Anderson KL, Itzhak Y. Adolescent and adult responsiveness to the incentive value of cocaine reward in mice: role of neuronal nitric oxide synthase (nNOS) gene. *Neuropharmacology*. 2006; 51:341–9. [PubMed: 16698049]
- Ben Mamou C, Gamache K, Nader K. NMDA receptors are critical for unleashing consolidated auditory fear memories. *Nat Neurosci*. 2006; 9:1237–1239. [PubMed: 16998481]
- Bhargava HN, Kumar S. Sensitization to the locomotor stimulant activity of cocaine is associated with increases in nitric oxide synthase activity in brain regions and spinal cord of mice. *Pharmacology*. 1997; 55:292–8. [PubMed: 9413858]
- Brenman JE, Bredt DS. Synaptic signaling by nitric oxide. *Curr Opin Neurobiol*. 1997; 3:374–378. [PubMed: 9232800]
- Chen Q, He S, Hu XL, Yu J, Zhou Y, Zheng J, Zhang S, Zhang C, Duan WH, Xiong ZQ. Differential roles of NR2A- and NR2B-containing NMDA receptors in activity-dependent brain-derived neurotrophic factor gene regulation and limbic epileptogenesis. *J Neurosci*. 2007; 27:542–552. [PubMed: 17234586]
- Chenard BL, Bordner J, Butler TW, Chambers LK, Collins MA, De Costa DL, Ducat MF, Dumont ML, Fox CB, Mena EE, Menniti FS, Nielsen J, Pagnozzi MJ, Richter KEG, Ronau RT, Shalaby IA, Stemple ZJ, White WF. (1S,2S)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol: a potent new neuroprotectant which blocks N-methyl-D-aspartate responses. *J Med Chem*. 1995; 38:3138–3145. [PubMed: 7636876]

- Christopherson KS, Hillier BJ, Lim WA, Bredt DS. PSD-95 assembles a ternary complex with the N-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. *J Biol Chem.* 1999; 274:27467–27473. [PubMed: 10488080]
- Collingridge G. Synaptic plasticity: the role of NMDA receptors in learning and memory. *Nature.* 1987; 330:604–605. [PubMed: 2825035]
- Conrad KL, Louderback KM, Milano EJ, Winder DG. Assessment of the impact of pattern of cocaine dosing schedule during conditioning and reconditioning on magnitude of cocaine CPP, extinction and reinstatement. *Psychopharmacology.* 2013; 227:109–116. [PubMed: 23269522]
- Cull-Candy SG, Leszkiewicz DN. Role of distinct NMDA receptor subtypes at central synapses. *Sci STKE.* 2004; 255:re16. [PubMed: 15494561]
- Furukawa H, Singh SK, Mancusso R, Gouaux E. Subunit arrangement and function in NMDA receptors. *Nature.* 2005; 438:185–192. [PubMed: 16281028]
- Huang YH, Lin Y, Mu P, Lee BR, Brown TE, Wayman G, Marie H, Liu W, Yan Z, Sorg BA, Schlüter OM, Zukin RS, Dong Y. In vivo cocaine experience generates silent synapses. *Neuron.* 2009; 63:40–47. [PubMed: 19607791]
- Hyman SE, Malenka RC, Nestler EJ. Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu Rev Neurosci.* 2006; 29:565–598. [PubMed: 16776597]
- Itzhak Y, Anderson KL. Changes in the magnitude of drug-unconditioned stimulus during conditioning modulate conditioned place preference in mice. *Addict Biol.* 2012; 17:706–716. [PubMed: 21507159]
- Itzhak Y, Anderson KL. Memory reconsolidation of cocaine-associated context required nitric oxide signaling. *Synapse.* 2007; 61:1002–1005. [PubMed: 17853433]
- Itzhak Y, Martin JL, Black MD, Huang PL. The role of neuronal nitric oxide synthase in cocaine-induced conditioned place preference. *Neuroreport.* 1998; 9:2485–2488. [PubMed: 9721919]
- Kelley JB, Anderson KL, Itzhak Y. Long-term memory of cocaine-associated context: disruption and reinstatement. *Neuroreport.* 2007; 18:777–780. [PubMed: 17471065]
- Kiraly DD, Lemtiri-Chlieh F, Levine ES, Mains RE, Eipper BA. Kalirin binds the NR2B subunit of the NMDA receptor, altering its synaptic localization and function. *J Neurosci.* 2011; 31:12554–12565. [PubMed: 21880917]
- Knackstedt LA, Kalivas PW. Extended access to cocaine self-administration enhances drug-primed reinstatement but not behavioral sensitization. *J Pharmacol Exp Ther.* 2007; 322:1103–1109. [PubMed: 17601982]
- Krapivinsky G, Krapivinsky L, Manasian Y, Ivanov A, Tyzio R, Pellegrino C, Ben-Ari Y, Clapham DE, Medina I. The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. *Neuron.* 2003; 40:775–784. [PubMed: 14622581]
- Lee MC, Yasuda R, Ehlers MD. Metaplasticity at single glutamatergic synapses. *Neuron.* 2010; 66:859–870. [PubMed: 20620872]
- Liddie S, Anderson KL, Paz A, Itzhak Y. The effect of phosphodiesterase inhibitors on the extinction of cocaine-induced conditioned place preference in mice. *J Psychopharmacol.* 2012; 26:1375–1382. [PubMed: 22596207]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and 2(-Delta Delta C(T)) Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
- Ma YY, Guo CY, Yu P, Lee DY, Han JS, Cui CL. The role of NR2B containing NMDA receptor in place preference conditioned with morphine and natural reinforcers in rats. *Exp Neurol.* 2006; 200:343–355. [PubMed: 16631172]
- Miller CA, Marshall JF. Molecular substrates for retrieval and reconsolidation of cocaine-associated contextual memory. *Neuron.* 2005; 47:873–884. [PubMed: 16157281]
- Milton AL, Lee JL, Butler VJ, Gardner R, Everitt BJ. Intra-amygdala and systemic antagonism of NMDA receptors prevents the reconsolidation of drug-associated memory and impairs subsequently both novel and previously acquired drug-seeking behaviors. *J Neurosci.* 2008; 28:8230–8237. [PubMed: 18701685]
- Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Development and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron.* 1994; 12:529–540. [PubMed: 7512349]

- Nie L, Wu G, Zhang W. Correlation of mRNA expression and protein abundance affected by multiple sequence features related to translation efficiency in *Desulfovibrio vulgaris*: a quantitative analysis. *Genetics*. 2006; 174:2229–2243. [PubMed: 17028312]
- Nutt JG, Gunzler SA, Kirchhoff T, Hogarth P, Weaver JL, Krams M, Jamerson B, Menniti FS, Landen JW. Effects of a NR2B selective NMDA glutamate antagonist, CP-101,606, on dyskinesia and Parkinsonism. *Mov Disord*. 2008; 23:1860–1866. [PubMed: 18759356]
- Ota KT, Pierre VJ, Ploski JE, Queen K, Schafe GE. The NO-cGMP-PKG signaling pathway regulates synaptic plasticity and fear memory reconsolidation in the lateral amygdala via activation of ERK/MAP kinase. *Learn Mem*. 2008; 15:792–805. [PubMed: 18832566]
- Perry JL, Morgan AD, Anker JJ, Dess NK, Carroll ME. Escalation of i.v. cocaine self-administration and reinstatement of cocaine-seeking behavior in rats bred for high and low saccharin intake. *Psychopharmacology*. 2006; 186:235–245. [PubMed: 16596398]
- Preskorn SH, Baker B, Kolluri S, Menniti FS, Krams M, Landen JW. An innovative design to establish proof of concept of the antidepressant effects of the NR2B subunit selective N-methyl-D-aspartate antagonist, CP-101,606, in patients with treatment-refractory major depressive disorder. *J Clin Psychopharmacol*. 2008; 28:631–637. [PubMed: 19011431]
- Sattler R, Xiong Z, Lu WY, Hafner M, MacDonald JF, Tymianski M. Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. *Science*. 1999; 284:1845–1848. [PubMed: 10364559]
- Sobczyk A, Scheuss V, Svoboda K. NMDA receptor subunit-dependent $[Ca^{2+}]$ signaling in individual hippocampal dendritic spines. *J Neurosci*. 2005; 25:6037–6046. [PubMed: 15987933]
- Suzuki T, Kato H, Tsuda M, Suzuki H, Misawa M. Effects of the non-competitive NMDA receptor antagonist ifenprodil on the morphine-induced place preference in mice. *Life Sci*. 1999; 64:151–156.
- Sweatt JD. The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *J Neurochem*. 2001; 76:1–10. [PubMed: 11145972]
- Torrás-García M, Lelong J, Tronel S, Sara SJ. Reconsolidation after remembering and odor-reward association requires NMDA receptors. *Learn Mem*. 2005; 12:18–22. [PubMed: 15647596]
- Tronson NC, Taylor JR. Addiction: a drug-induced disorder of memory reconsolidation. *Curr Opin Neurobiol*. 2013; 4:573–580. [PubMed: 23415831]
- Valjent E, Corbillé AG, Bertran-Gonzalez J, Hervé D, Girault JA. Inhibition of ERK pathway or protein synthesis during reexposure to drugs of abuse erases previously learned place preference. *Proc Natl Acad Sci USA*. 2006; 103:2932–2937. [PubMed: 16473939]
- Wang D, Cui Z, Zeng Q, Kuang H, Wang LP, et al. Genetic enhancement of memory and long-term potentiation but not CA1 long-term depression in NR2B transgenic rats. *PLoS One*. 2009; 4:e7486. [PubMed: 19838302]
- White NM, Carr GD. The conditioned place preference is affected by two independent reinforcement processes. *Pharmacol Biochem Behav*. 1985; 23:37–42. [PubMed: 2994120]
- Yashiro K, Philpot BD. Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity. *Neuropsychopharmacology*. 2008; 55:1081–1094.
- Zhao MG, Toyoda H, Lee YS, Wu LJ, Ko SW, Zhang XH, Jia Y, Shum F, Xu H, Li BM, Kaang BK, Zhuo M. Roles of NMDA NR2B subtype receptor in prefrontal long-term potentiation and contextual fear memory. *Neuron*. 2005; 47:859–872. [PubMed: 16157280]
- Zhuo M. Plasticity of NMDA receptor NR2B subunit in memory and chronic pain. *Mol Brain*. 2009; 3:4. [PubMed: 19192303]

Fig. 1. The NR2B subunit of NMDA receptor contributes to the development of Esc-C memory (A) Mice that received cocaine paired to the training context develops CPP. Data are represented as mean $\pm$ SEM of difference in time (sec) spent on cocaine- vs. saline-paired compartment. (* $p < 0.001$), difference between Pre-CPP and Post-CPP (CPP Test); (# $p < 0.05$) difference between the paired groups of Fix-C and Esc-C. For Fix-C: saline (n=13); unpaired (n=4); paired (n=10). For Esc-C: saline (n=13); unpaired (n=8); paired (n=9). Panel B pertains to paired and unpaired groups while panels C, D and E pertain only to paired groups. (B) RT-qPCR analysis showed that Grin2b mRNA (which codes for NR2B protein) was significantly up-regulated in Esc-C compared to all other groups. (C) Mice conditioned by Esc-C had significantly elevated levels of NR2B protein compared to Fix-C and saline-treated mice. (D) Expression of NR1 protein was unchanged across conditioning groups. (E) Changes in NR2A subunit expression were not statistically significant. * $p < 0.05$ and ** $p < 0.01$. For western blots n=3–4/group and for qPCR n=4/group.

Fig. 2. Acquisition of cocaine memory: antagonism of NR2B-containing NMDA receptors attenuates the acquisition of both Fix-C and Esc-C memory while nNOS inhibition attenuates Fix-C but not Esc-C memory acquisition

(A) Schematic of experimental procedure. (B, C) The NR2B-containing NMDAR antagonist ifenprodil (10mg/kg) significantly reduces the development of place preference following conditioning by Fix-C and Esc-C. (D, E) The nNOS inhibitor 7-NI (25mg/kg) significantly reduces the development of CPP following conditioning by Fix-C but not Esc-C schedule. Data are presented as mean $\pm$ SEM of difference in time spent on cocaine- vs saline-paired compartment (sec). (n=6–10/group; *p<0.05).

Fig. 3. Reconsolidation of cocaine memory: temporal- and dose-dependence of NMDAR channel blockade

(A) Schematic of experimental procedure. (B) Administration of MK-801 prior to memory retrieval inhibits extinction learning in mice conditioned by Fix-C. (C) Pre-retrieval administration of MK-801 had no effect on Esc-C memory. (D) Post-retrieval administration of low dose MK-801 (0.1mg/kg) disrupts Fix-C memory reconsolidation. (E) Post-retrieval administration of high (0.3mg/kg; n=10) but not low (0.1mg/kg; n=7) dose of MK-801 disrupts subsequent expression of CPP in the Esc-C group compared to saline (n=8). Data are presented as mean $\pm$ SEM of difference in time spent on cocaine-vs saline-paired compartment (sec). * $p < 0.05$.

Fig. 4. Reconsolidation of cocaine memory: antagonism of NR2B-containing NMDARs disrupts Fix-C and Esc-C memory reconsolidation

(A) Schematic of experimental procedure. (B, C) Immediate post-retrieval administration of ifenprodil (10mg/kg) significantly attenuates Fix-C and Esc-C place preference expression. (D) Administration of ifenprodil (10mg/kg) to Esc-C conditioned mice either in the home cage or 6 hr after memory retrieval (outside reconsolidation window) did not reduce subsequent CPP expression. (E, F) The NR2B-containing NMDA receptors antagonist traxoprodil (10mg/kg) disrupts both Fix-C and Esc-C memory reconsolidation. Data are presented as mean $\pm$ SEM of difference in time spent on cocaine-vs saline-paired compartment. *p<0.05.

Fig. 5. Effect of inhibition of NMDAR downstream signaling on reconsolidation of cocaine memory

Fix-C memory reconsolidation is NO-dependent but MEK-independent while Esc-C memory reconsolidation is MEK-dependent and NO-independent. (A) Schematic of experimental procedure. (B, C) Administration of the nNOS inhibitor 7-NI (25mg/kg) immediately following memory retrieval attenuates subsequent place preference acquired by Fix-C but not Esc-C schedule. (D, E) Post-retrieval administration of the MEK inhibitor SL327 (30mg/kg) attenuates subsequent place preference acquired by Esc-C but not Fix-C schedule. Data are presented as mean $\pm$ SEM of difference in time spent on cocaine-vs saline-paired compartment. *p<0.05.